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p-Nonyl-Phenol: An Estrogenic Xenobiotic Released from "Modified" Polystyrene

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Alkylphenols are widely used as plastic additives and surfactants. We report the identification of an alkylphenol, nonylphenol, as an estrogenic substance released from plastic centrifuge tubes. This compound was extracted with methanol, purified by flash chromatography and reverse-phase high performance liquid chromatography, and identified by gas chromatography-mass spectrometry. Nonylphenol induced both cell proliferation and progesterone receptor in human estrogen-sensitive MCF_7 breast tumor cells. Nonylphenol also triggered mitotic activity in rat endometrium; this result confirms the reliability of the MCF_7 cell proliferation bioassay. The estrogenic properties of alkylphenols, specifically nonylphenols, indicate that the use of plasticware containing these chemicals in experimental and diagnostic tests may lead to spurious results, and these compounds as well as alkylphenol polyethoxylates may also be potentially harmful to exposed humans and the environment at large.

Introduction

Estrogens regulate cell proliferation and gene expression in the female genital tract, pituitary, and mammary gland (*1–3*). Induction of mitotic activity in the female genital tract has been considered the hallmark of estrogen action (*1*), and estrogen bioassays were designed to measure this proliferative event, albeit in an indirect fashion [i.e., the Allen Doisy assay measuring vaginal cornification (*4*)]. The proliferative effect of estrogens is readily apparent in established estrogen-sensitive cells in culture; these models closely reproduce the effects observed *in situ* (*5–7*). In addition to steroid hormones, nonsteroidal substances of widely diverse chemical structure mimic estrogen action (*8,9*). This diversity makes it difficult to predict the estrogenicity of xenobiotics solely on structural bases (*10*). Thus, it is not surprising that the estrogenic properties of most xenobiotics are discovered after their release into the environment (*9*); in fact, the estrogenic properties of the pesticide chlordecone were inferred from clinical observations on workers at a chlordecone manufacturing plant (*11*). The estrogenicity of this and other pesticides was later confirmed in animal models (*9,12*).

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Phytoestrogens (*13*) and mycoestrogens (*14*) present in animal feeds have been shown also to cause reproductive impairment in domesticated species and laboratory rodents. We report here that polystyrene tubes used in routine laboratory procedures release a substance with estrogenic properties. This substance was identified as a nonylphenol. This and other alkylphenols are used as antioxidants in the plastic industry (*15*) and have been reported to leach from plastics used in food processing and packing (*16*).

Materials and Methods

Cell Lines and Culture Conditions

Human breast tumor MCF_7 cells (*17*) were originally obtained from C. McGrath, Michigan Cancer Foundation; they were cloned in our laboratory (*6*). Cells were maintained routinely in Dulbecco's modification of Eagle medium (DME) (GIBCO, Grand Island, New York) supplemented with 5% fetal bovine serum (FBS) from Hyclone (Logan, Utah). Cells were routinely grown in 75 cm^2 plastic flasks (Corning Plastic, Corning, New York).

Cell Proliferation Experiments

Charcoal-dextran stripped (CD) human serum (HuS) inhibits the proliferation of MCF_7 cells in a dose-dependent fashion (*6*). Estrogens overcome this inhibitory effect (*6,7*). This is the underlying principle for the

bioassay used to characterize and purify a discrete inhibitory factor present in human serum and to assess the proliferative effect of estrogens (*18*). Human serum was obtained from young male and female adult, healthy volunteers; endogenous estrogens were removed by charcoal-dextran stripping (*6*). All cell proliferation experiments were carried out in media free of phenol red (*19*).

Cell proliferation yields were determined by seeding cells in Costar 3512 multiwell plates (Cambridge, MA) in 1 mL 5% FBS-supplemented DME for 24 hr so cells would attach to the plastic surface; seeding medium was changed to 0.9 mL/well of 10% charcoal-dextran stripped human serum (CDHuS)-supplemented DME (*6*). Estradiol-17β (E_2) and substances suspected of being estrogenic were dissolved in 10% CDHuS to desired concentration, and 0.1 mL of these solutions was added to each well. After 6 days, cells were lysed (10% Zapoglobin [Coulter Electronics, Hialeah, Florida]), and nuclei were counted on a Coulter Counter Model Zf_i (*6*).

Characterization and Purification Procedures

CDHuS stored in newly purchased polystyrene tubes (Corning Glass Co., Cat. # 25310-15) no longer inhibited MCF_7 cell proliferation. We learned from the manufacturer that these tubes were made with a "modified" polystyrene formulation that was more resistant to breakage due to centrifugation than the conventional polystyrene tubes. For the sake of clarity, we call "modified" polystyrene the one used by Corning to make those tubes, and "unmodified" polystyrene that used by Falcon Plastics to make their tubes under Cat. # 2095. Modified and unmodified polystyrene tubes were extracted with 5 mL methanol/tube for 2 hr at 37°C in a roller apparatus (6 cycles/min); this extract was dried under a nitrogen stream. The residue was resuspended in hexane and chromatographed through a 4 × 220 mm silica column (ABI, Santa Clara, CA) equilibrated with *n*-hexane. Elution was carried out isocratically at a flow rate of 1.5 mL/min; the elution profile was monitored at 278 nm. Column eluates were dried down, resuspended in 10% CDHuS, made sterile by filtration, and added to MCF_7 cells as described above.

Preparative Purification Procedure

Fifty modified polystyrene tubes were extracted as indicated above, the extract was evaporated under N_2, resuspended in chloroform, and flash chromatographed through a 20-mL silica grade 60 column (25 cm × 1.5 cm; 6 nm pore size, Aldrich Chemical Co., Milwaukee, Wisconsin) equilibrated with chloroform. Elution was monitored by thin layer chromatography (rf = 0.30 in $CHCl_3$); bioactivity was assessed by measuring the proliferation yield of MCF_7 cells. The active peak was dried under N_2, dissolved in a 75:25 methanol:water mixture, prepared for high performance liquid chromatography by elution through a C18 Sep-PAK cartridge (Millipore Co, Bedford, Massachusetts), and chromatographed through a C8 reverse-phase column (1 × 20 cm, 10 μm particle size, ABI, Santa Clara, California). Chromatographic resolution was optimized empirically by maximizing separation of alkylphenols of different alkyl chain lengths (Aldrich Chemical Co., Milwaukee, Wisconsin). The column was equilibrated in 50% methanol-water (solvent A), and eluted with 100% methanol (solvent b) in a complex gradient (50–90% B in 5 min; 90–100% B in 20 min) with a flow rate of 3 mL/min. Moieties separated by C8 reverse-phase chromatography were dried down, weighed, resuspended in ethanol, and diluted in 10% CDHuS. To assess estrogenicity, 0.1 mL of the appropriate stock solution was added to each well containing 0.9 mL of 10% CDHuS.

Spectroscopic Studies

Electron-impact mass spectra of polystyrene-derived alkylphenol and technical grade nonylphenol (a mixture of nonylphenols of branched nonyl chains, > 85% *p*-substituted; Fluka Chemical Corp., Rokonkoma, New York) were obtained on a HP5985 gas chromatography/mass spectrometry (GC/MS) system operating at an ion source temperature of 200°C. The temperature of the GC injector and transfer line was 100°C. A fused silica column (3 m × 2 mm) was used with argon as the carrier gas. Column temperature was held at 70°C, then programmed from 70 to 270°C at a rate of 10°C/min. High resolution MS was performed in a Varian MAT 731 double-focusing mass spectrometer operated at a resolution 1:14,000. Spectra were obtained using electron ionization. Sample ions were peak-matched; the reference compound was perfluorokerosene. Proton nuclear magnetic resonance spectroscopy was performed in a Varian XL 300 NMR; infrared spectroscopy was performed in a Nicolet 510 FT-IR spectrometer.

Progesterone Receptor Induction

Duplicate plates were seeded as described for cell yield experiments (see above). After 6 days of exposure to test substances, one plate was harvested for cell counting, and the other was processed for progesterone receptor (PgR) determination as follows: culture media were aspirated, attached cells were gently rinsed *in situ* with 1 mL PBS, and then plates were frozen at –80°C for 18 hr. PgR was extracted by incubation with 0.2 mL of 0.5 M KCl, 10 mM Tris, 1.5 mM EDTA, 5 mM sodium molybdate, 1 mM monothioglycerol, pH 7.4, at 4°C for 1 hr (*20*). Cell extracts were clarified by centrifugation in an Eppendorf centrifuge. PgR levels were determined using the Abbott EIA (Abbott, Diagnostic Division, North Chicago, Illinois) following the manufacturer's protocol. Briefly, the cell extracts were incubated at 4°C for 18 hr with a rat anti-human PgR monoclonal antibody that is covalently attached to beads, the beads were washed and incubated with horseradish peroxi-

dase-coupled rat anti-human PgR antibody for 1 hr at 4°C, and beads were washed again and incubated with substrate (O-phenelyenediamine) for 30 min. The reaction was stopped with 1 N sulfuric acid and read at 492 nm.

Animal Experiments

Adult Sprague-Dawley female rats weighing 120 to 150 g were ovariectomized and primed with 15 ng E_2 at 12, 13, and 14 days after ovariectomy (*21*). Doses ranging from 1 to 50 mg of the polystyrene-derived purified alkylphenol (peak #2, C8 reverse-phase column) dissolved in 0.1 mL sesame oil were administered SC 19 and 20 days after ovariectomy. Positive controls were injected with 1.25 μg E_2, and negative controls were treated with vehicle only. Animals were killed by exposure to CO_2 24 hr after the second injection. Colchicine (2 mg/kg body weight) was administered IP 4 hr before sacrifice to arrest endometrial mitotic figures and assess mitotic indices. Statistical treatment was performed by analysis of variance followed by the Newman-Keuls test (*22*).

Results

Cell Proliferation Experiments

MCF_7 cells proliferated maximally in medium supplemented with CDHuS stored in modified polystyrene tubes. To the contrary, cells supplemented with CDHuS stored in glass, polypropylene or plain (unmodified) polystyrene tubes required E_2 supplementation for maximal proliferation yields (Fig. 1). To characterize the substance responsible for this unexpected proliferative effect, methanol extracts from both "unmodified" and "modified" polystyrene tubes were purified by normal phase HPLC (Fig. 2*A*); the separated components were tested for their ability to induce MCF_7 cell proliferation (Fig. 2*B*). The modified polystyrene extract contained a single active fraction absent in the unmodified polystyrene extract (peak #2, retention time: 4 min). The active fraction reacted with diazo *p*-nitroaniline to form a colored product suggesting that it contained a phenol (*23*).

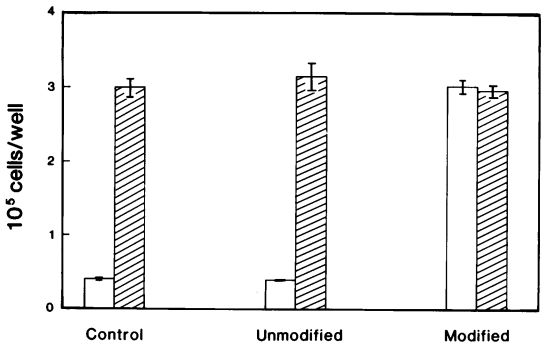

FIGURE 1. Five-milliliter aliquots of CDHuS were incubated for 1 hr at 37°C in 15 mL tubes made of unmodified and modified polystyrene. Proliferation yields were measured in the presence (hatched bars) and absence of 30 pM E_2 (open bars) in triplicate cultures. Cells were harvested and counted 6 days after exposure to CDHuS. Brackets indicate standard deviations.

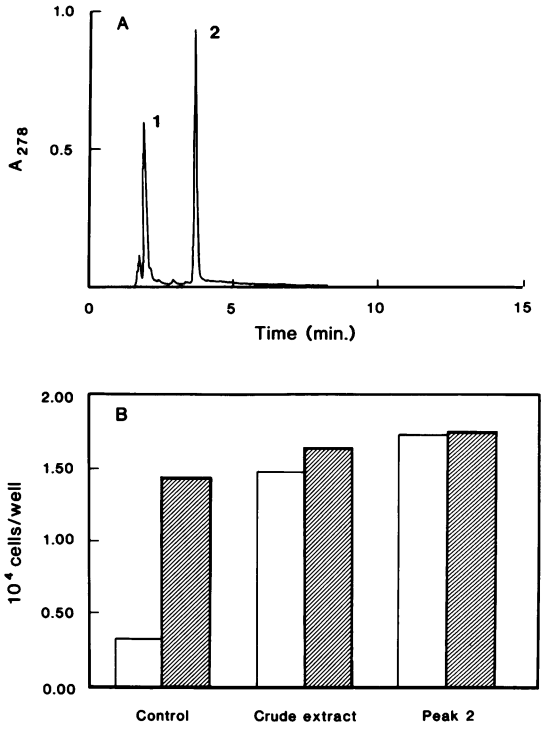

FIGURE 2. Separation by normal phase chromatography. (*A*) Modified polystyrene extract was resolved in 2 peaks (retention times: peak 1=1.9 min and peak 2 = 4.0 min). Unmodified polystyrene extract was resolved in a single peak similar to peak 1 of the modified polystyrene extract and coeluted with styrene monomer. (*B*) Proliferative effect of unmodified polystyrene extract (control), modified polystyrene extract (crude extract), and peak 2 of the chromatogram shown in panel *A*. MCF_7 cell yields obtained in medium supplemented with 10% CDHuS. Open bars: extracts were obtained with 5 mL methanol/tube, dried down and resuspended in 1 mL 10% CDHuS; 0.1 mL of this solution was added to each well. Peak 2 obtained from 5 mL 10% CDHuS; 0.1 mL was added to each well. Hatched bars: similar inocula were treated as indicated above; in addition, 30 pM E_2 was added. Each bar represents the mean of triplicate cultures. Standard deviations were within 5% of the mean.

Purification of the Putative Estrogenic Contaminant

The flash chromatography fraction having an estrogenlike effect on MCF_7 cell proliferation was further purified by chromatography in a C18 Sep-Pak cartridge and HPLC in a C8 column. Three peaks were resolved (Fig. 3*A*); they were dried down, dissolved in 10% CDHuS and tested for proliferative effects in culture. They all induced proliferation of MCF_7 cells (Fig. 3*B*).

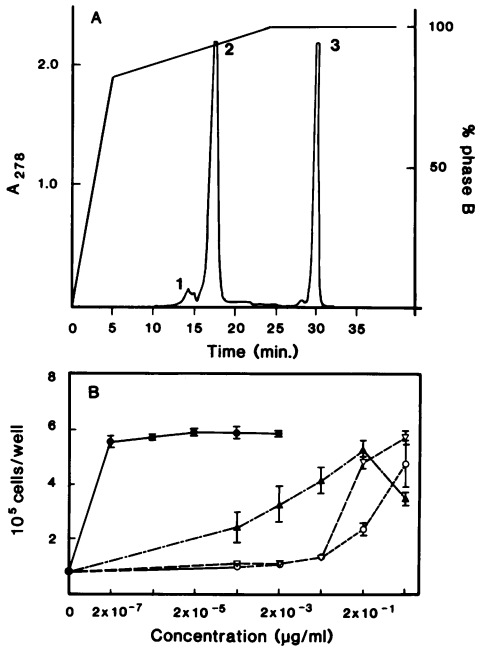

FIGURE 3. (*A*) Elution profile of modified polystyrene-derived alkylphenols by reverse phase chromatography in a C8 column. (*B*) Proliferative pattern of MCF_7 cells harvested after 6 days of exposure to the putative estrogen. Peak 1 (○--○), peak 2 (△--△), peak 3 (▽--▽), E_2 (●—●). Result points represent the mean of triplicate cultures; brackets represent standard deviations.

Spectroscopic Studies

Peak #2 from the C8 column was analyzed by infrared spectroscopy (IR), proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), and GC/MS. The IR spectrum revealed an alcohol with a *para*-substituted aromatic ring. ^{1}H-NMR spectroscopy confirmed the IR findings revealing aromatic protons at 7.3 and 6.9 ppm, the phenyl alcohol proton at 4.8 ppm and protons of the long aliphatic chain between 0.8 and 2.0 ppm. Lack of proton signals between 2 and 4 ppm indicated no protons alpha to the aromatic ring. Further characterization was obtained by GC/MS (Fig. 4*A*). The molecular ion has a mass of 220. The spectra also revealed a fragment ion consistent with the loss of terminal C_2H_5 at m/e = 191, a fragment ion at m/e = 177, 163, 149, and 135 consistent with serial fragmentation by loss of CH_2. The elemental composition of the molecular ion and the m/E = 191 fragment ion were ascertained by high resolution MS (molecular ion measured value 220.1828, calculated value 220.1827, empirical formula $C_{15}H_{24}O$; fragment ion measured value 191.1447, calculated value 191.1436, empirical formula $C_{13}H_{19}O$). The most probable structure is a *para*-substituted nonylphenol. Technical grade nonylphenol, a mixture of isomeric compounds with differently branched structures of the nonyl side chains (85% is *p*-nonyl phenol) was analyzed by GC/MS; the spectra of each one of the separated peaks contained all fragments present in our purified alkylphenol. Figure 4*B* shows the spectrum of one of the moieties of this mixture; abundance of fragments here most closely resemble

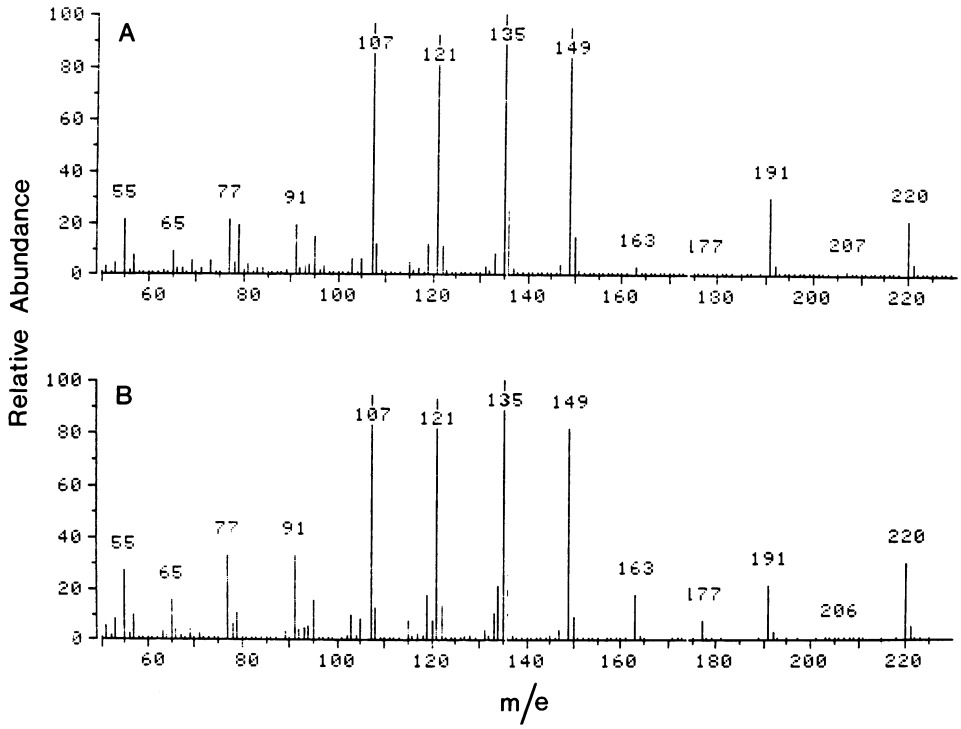

FIGURE 4. Electron-impact mass spectra of (*A*) plastic-derived alkylphenol and (*B*) technical-grade nonylphenol.

those in the polystyrene-derived alkylphenol. A comparable fragmentation pattern was indicative of an α-ethyl-α-methyl-substituted nonyl side chain (*24*). Industrial grade nonylphenol was as active as the polystyrene-derived compound to induced MCF_7 cell proliferation (Table 1). *Ortho-*, *meta-*, and *para*-substituted *tert*-butylphenols were tested for estrogenicity; only *p-tert*-butylphenol was active (Fig. 5).

Animal Experiments

The estrogenlike activity of the modified polystyrene-derived alkylphenol was tested in ovariectomized rats. The endometrial mitotic index induced by 50 mg of the alkylphenol purified from modified polystyrene tubes was significantly higher than the one observed in ovariectomized animals ($p < 0.05$) and lower than the one observed with E_2 ($p < 0.05$) (Fig. 6). A 20-mg dose induced a weak proliferative response while the mitotic indices at doses below 10 mg were similar to those found in control animals.

Table 1. Proliferative response of MCF_7 cells to polystyrene-derived nonylphenol and *p*-nonylphenol, industrial grade.[a]

Compound	Concentration	Cell number
No additions		152,030 ± 3,119
Estradiol	3 pM	316,490 ± 10,461
	30 pM	718,850 ± 25,278
Polystyrene-derived	100 nM	148,480 ± 2,017
nonylphenol	1 μM	253,320 ± 18,514
	10 μM	703,240 ± 15,490
p-Nonylphenol, Fluka	100 nM	145,250 ± 7,446
	1 μM	194,520 ± 6,661
	10 μM	724,620 ± 13,715

[a]Proliferative yield of MCF_7 cells was measured after 6 days of exposure to the putative estrogen. Results represent the mean of triplicate cultures ± standard deviations.

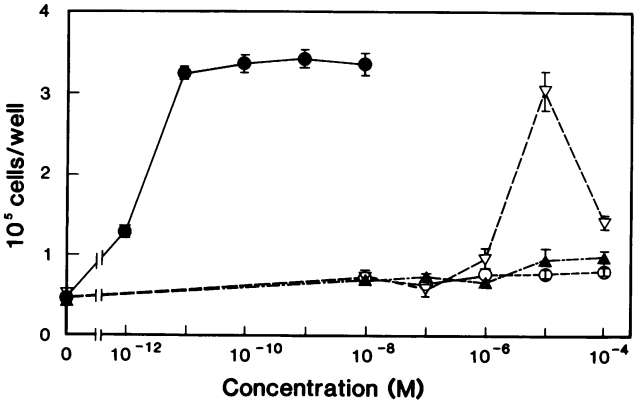

FIGURE 5. MCF_7 cell yield experiment comparing the effect of *ortho* (○--○), *meta* (▲--▲); and *para* (▽--▽) substitutions on the estrogenic potency of *tert*-butylphenol. A dose-response curve to E_2 was run simultaneously (●—●). Result points represent triplicate cultures; brackets indicate standard deviations.

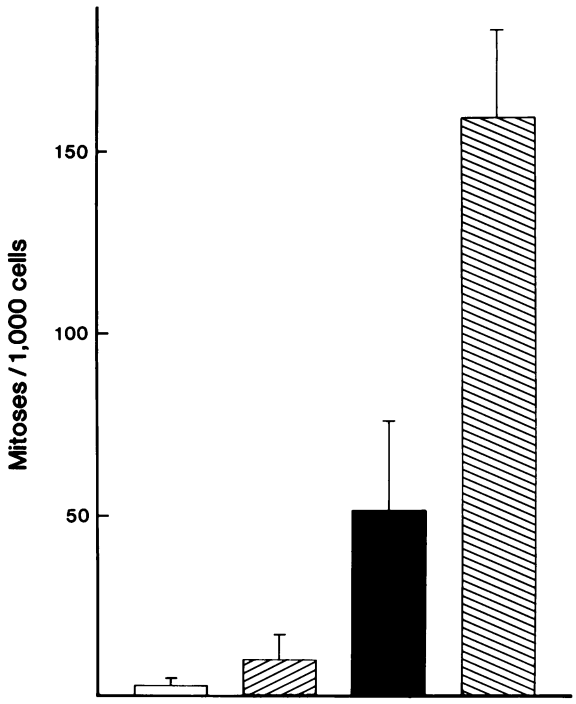

FIGURE 6. Estrogenic activity of the modified polystyrene-derived alkylphenol (peak 2 from the C8 reverse phase column) was assessed by comparing the mitotic index of luminal endometrial epithelium of ovariectomized animals treated with vehicle (open bar), 20 mg alkylphenol (left hatched bar), 50 mg alkylphenol (solid bar), and 1.25 μg E_2 (right hatched bar).

Induction of Progesterone Receptor

PgR is a widely accepted marker for functionality of the estrogen receptor pathway (*5,20*). The *p*-nonylphenol extracted from modified polystyrene was equally effective at inducing cell proliferation and PgR in MCF_7 cells (Fig. 7).

Discussion

Alkylphenols are used as antioxidants in the manufacture of plastics such as polyvinyl chloride (PVC) and polystyrene (*15*); this explains the presence of alkylphenol in modified polystyrene centrifuge tubes. *p*-Nonylphenol, the alkylphenol identified as the substance leaching from the plastic centrifuge tubes used in our laboratory, has estrogenlike properties when tested in the human breast tumor MCF_7 cell line and in the castrated rat models; the parameters used were cell proliferation and progesterone receptor induction. So far, substances that meet Hertz's definition of estrogenicity ["the primary effect of an estrogen is the stimulation of mitotic activity in the tissues of the female genital tract. A substance which can directly elicit this response is an estrogen; one that cannot is not" (*25*)] also induce cell proliferation in the MCF_7 model. Moreover, the proliferative effect of *p*-nonylphenol appears to be specific because only estrogens induce proliferation of

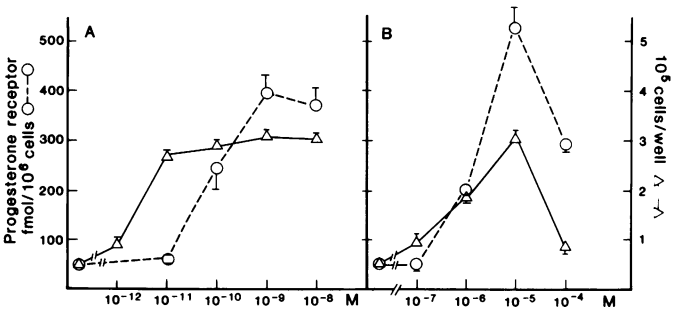

FIGURE 7. Proliferative response (△—△) and progesterone receptor induction (○--○) by E_2 (*A*) and polystyrene-derived alkylphenol (*B*) on MCF_7 cells in culture. Data points represent the mean of triplicate cultures; the brackets indicate standard deviations.

these cells as long as the medium in which they are tested contains serum rendered estrogenless by charcoal-dextran treatment (*5–7*). Also, the fact that the cells used in this bioassay are of human origin obviates the uncertainty faced when extrapolating results form experimental animals to medical applications. This model may be used to advantage for screening xenobiotics suspected of being estrogenic.

Data presented here have bearings upon other related issues. First, the definition of estrogenicity should be construed on biological rather than structural bases since no common feature has been found so far among all substances able to evoke estrogenic responses in animal models. For example, phenols are found in most but not all estrogenic xenobiotics (*10*). Following Hertz's definition (quoted above), we demonstrated that the alkylphenol extracted for modified polystyrene is indeed an estrogen in a rodent model (Fig. 6) and in the human breast cancer MCF_7 cell model, i.e., it induced both cell proliferation and progesterone receptor (Figs. 1, 3*B*, 5, and 7)

Second, are alkylphenols a likely cause of environmental hazard due to their estrogenicity? Alkylphenol was found in PVC used in the food processing and packaging industries (*26*). Nonylphenol may leach from plastic and was reported to contaminate water flowing through PVC tubing (*16*). The annual production of *p*-nonylphenol in the U.S. was 45,000 tons in 1976 (*27*). Nonylphenols are also used in the synthesis of surfactants such as nonoxyphenol, a compound present in intravaginal spermicides (*28*) and alkylphenol polyethoxylates used as industrial detergents (*29*). The annual production of alkylphenol polyethoxylates in the U.S. was 140,000 metric tons in 1982 (*29*). Although these detergents are unlikely to have estrogenic activity, they are known to be degraded to free nonylphenol in rodent models (*3*) as well as in sewage sludge (*31*). Anaerobically stabilized sludge samples were reported to contain 0.45 to 2.53 g *p*-nonylphenol/kg (*31*).

The relatively low estrogenic potency of these compounds does not rule out their potential toxicity after chronic exposure to animals or human beings. Fish in the Detroit river's Trenton Channel, nearby a chemical plant manufacturing alkylphenols, were reported to contain 40 μg of *p-tert*-pentylphenol per gram of fat tissue, a concentration higher than the ones found in the river sediment (*32*). The *p-tert*-pentylphenol concentration in carp adipose tissue was comparable to the alkylphenol concentration eliciting maximal cell proliferation in MCF_7 cells. This compound causes vaginal cornification in ovariectomized rats (*33*) and interacts with estrogen receptors (*34*). The bioaccumulative properties of alkylphenols (*32*) parallel those of chlordecone, which is sequestered in liver and adipose tissue, eliciting considerable estrogenic activity in spite of its low potency when compared to E_2 (*8,35*). In fact, reproductive effects such as oligospermia and sterility were reported in workers exposed to chlordecone (*11*). Estrogenic effects of alkylphenols and chlordecone in MCF_7 cells occur at comparable doses (unpublished data); this suggests that alkylphenols constitute health hazards to humans other animals when target cells *in situ* become exposed to micromolar levels of these compounds. Pharmacokinetic studies in animal models are needed to further assess the likelihood of human intoxication.

In conclusion, a bioassay using human estrogen-sensitive cells revealed estrogenlike effects of nonylphenol present in commercially available plastic centrifuge tubes. These effects were verified in rat endometrium as well. The estrogenic properties of nonylphenol and other alkylphenols may introduce unsuspected sources of error in experimental and diagnostic procedures designed to quantitate estrogens (radioimmunoassays, receptor assays, etc.). Also, alkylphenols are potentially harmful to the reproductive function of exposed humans and animals. The sizable alkylphenol production volume, the diversity of its uses, and its low biodegradability are factors that increase the likelihood of their presence in the food chain.

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